A novel multiplex bead-based platform highlights the diversity of extracellular vesicles

Nina Koliha[1], Yvonne Wiencek[1], Ute Heider[1], Christian Jüngst[2], Nikolay Kladt[2], Susanne Krauthäuser[1], Ian C. D. Johnston[1], Andreas Bosio[1], Astrid Schauss[2], Stefan Wild[1*]

[1]Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
[2]Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), Cologne, Germany

Supplementary Material

Supplementary methods:

Cell stainings and flow cytometry analysis

For cell analysis, 5x10^5 cells were stained in PEB (PBS with 0.5% BSA and 2 mM EDTA) with CD19-PE-Vio770 and CD20-PE, or CD80-PE-Vio770, CD20-PE, and CD86-VioBlue, or CD20-PE and CD42a-APC-Vio770, or with the respective isotype controls (Miltenyi Biotec) in final antibody dilutions of 1:11 with FcR Blocking reagent (Miltenyi Biotec) in a total volume of 110 µl for 10 min at 4°C. After washing with PEB, cells were resuspended in 100 µl PEB, dead cells were stained with PI (Propidium iodide) and the sample was analyzed by MACSQuant Analyzer 10 (Miltenyi Biotec). At least 10,000 events were measured per sample. In the forward/side scatter, debris was excluded from the analysis. Dead cells were identified by staining with propidium iodide and excluded. Gates were set considering the stainings with isotype control antibodies. For evaluation, MACSQuantify software version 2.6 (Miltenyi Biotec) was used.

Nanoparticle Tracking Analysis (NTA)

Size distribution of EVs were measured with a Nano Sight LM10 instrument equipped with a 532nm laser and an EMCCD camera (Malvern Instruments Ltd, Malvern, UK). For data analysis, NTA software version 3.1 was used. All measurements were

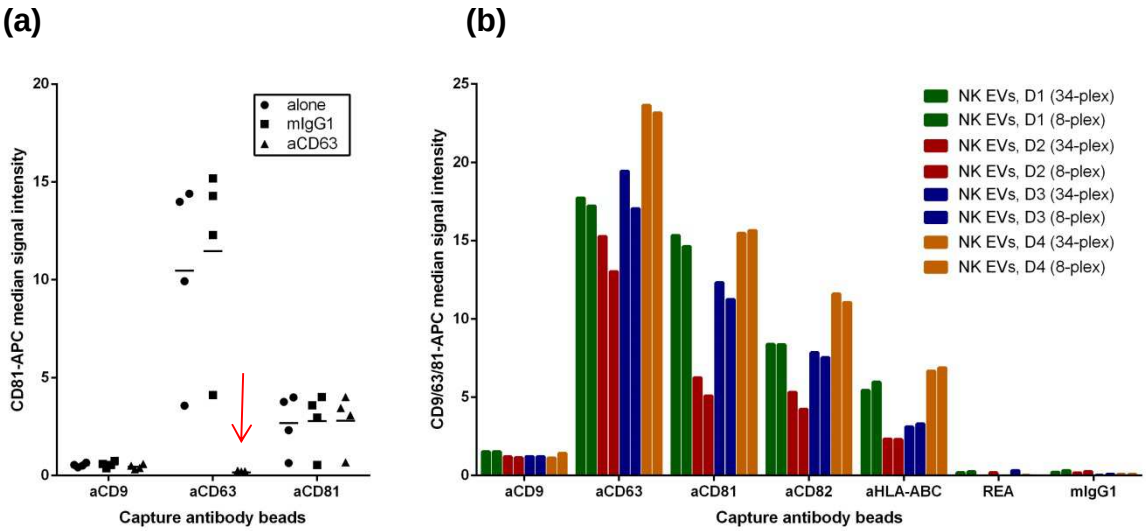
carried out with optimized settings from the same experienced operator to achieve comparable results.

Western Blot

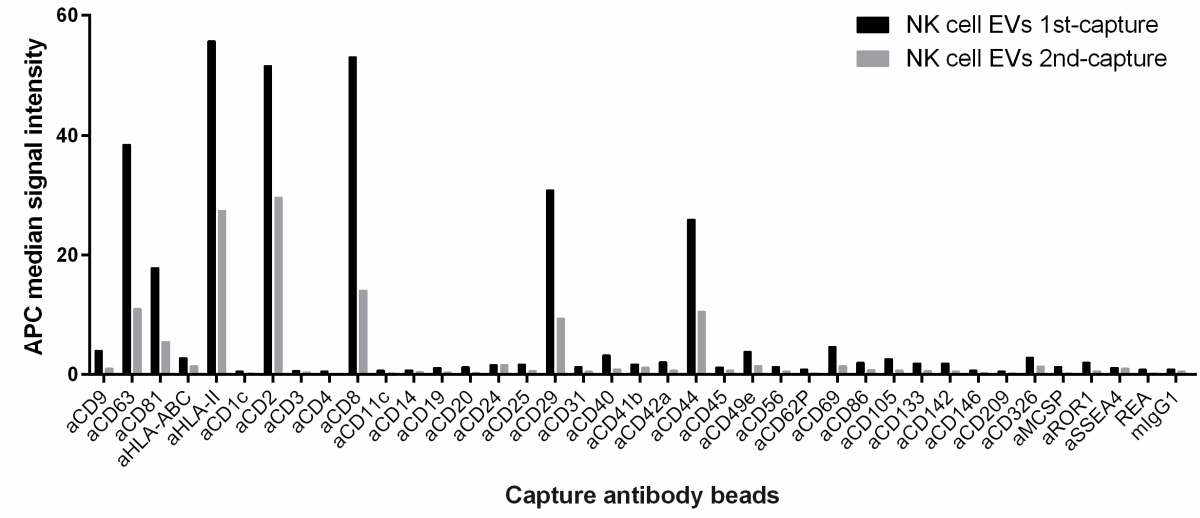
The EVs/bead eluates were concentrated using the Eppendorf Concentrator 5301 to about 45 µl. Samples were heated for 5 min at 95°C, 20 µl were loaded onto a SDS polyacrylamide gel (4-20% Tris Glycine gels, 1x SDS-running buffer) (anamed Elektrophorese GmbH) and proteins were separated at 220 V for 1 h 15 min. Gel and nitrocellulose membrane (Protran Premium 0.45 NC, (GE Healthcare) were equilibrated in transfer buffer (48 mM Tris, 39 mM Glycine, 1.3 mM SDS, 10% methanol) for 20 min. Proteins were blotted using the Biometra Fastblot B33 device (blotting parameters: 20 V, 100 mA/gel, 40 min) (Biometra GmbH). The membrane was incubated in blocking solution (5% nonfat dried milk powder in TBS (150 mM NaCl, 50 mM Tris pH 8.0) with 0.1% Tween 20) overnight at 4°C. The membrane was incubated with biotin-coupled antibodies (4.9 µg/ml CD9, clone SN4 (Miltenyi Biotec), 1 µg/ml CD81, clone 1.3.3.22 (Biomol GmbH) or 1 µg/ml CD29, clone TS2/16 (Miltenyi Biotec)) diluted in blocking solution for 1.5 h at room temperature. The membrane was washed four times for 5 min in TBS + 0.1% Tween 20. After the washing steps, the membrane was incubated with Streptavidin coupled to peroxidase (1:15000 in blocking solution) (Roche, Sigma-Aldrich Chemie GmbH) for 1.5 h and subsequently washed again four times for 5 min in TBS + 0.1% Tween 20. The signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and ECL Hyperfilm (GE Healthcare).

Supplementary Table 1: Overview of the used capture antibodies on the capture antibody beads

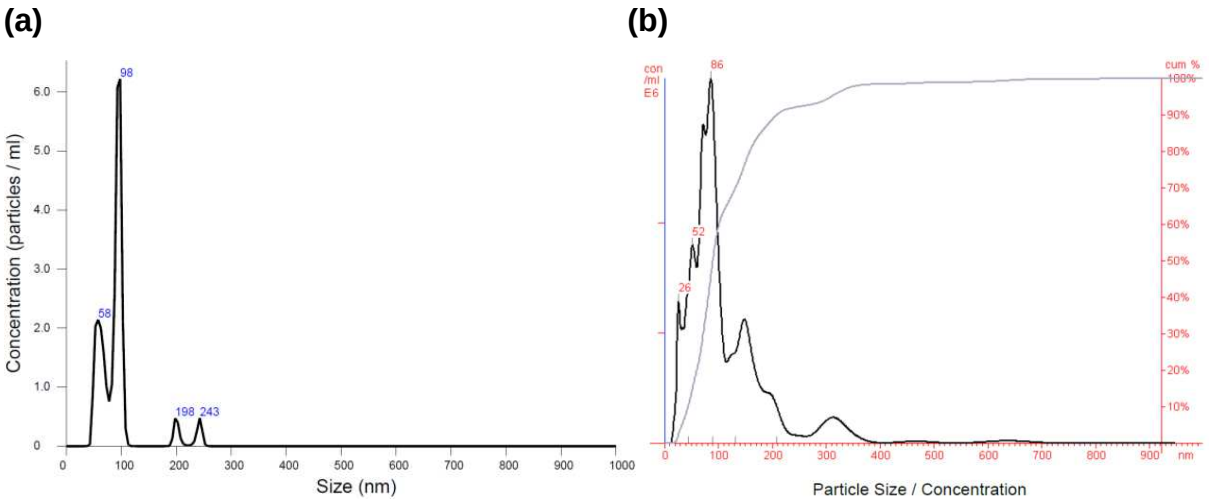
	Capture antibody	Clone	Company
1	CD9	SN4	Miltenyi Biotec
2	CD63	H5C6	Miltenyi Biotec
3	CD81	5A6	BioLegend
4	CD82	REA221	Miltenyi Biotec
5	HLA-ABC	REA230	Miltenyi Biotec
6	HLA-DQ	REA303	Miltenyi Biotec
7	HLA-DP/DQ/DR	REA332	Miltenyi Biotec
8	CD2	LT2.2	Miltenyi Biotec
9	CD11a	REA378	Miltenyi Biotec
10	CD11c	MJ4-27G12	Miltenyi Biotec
11	CD3	BW 264/56	Miltenyi Biotec
12	CD4	Vit-4.3	Miltenyi Biotec
13	CD8	BW 135/80	Miltenyi Biotec
14	CD14	TÜK4	Miltenyi Biotec
15	CD19	LT-19	Miltenyi Biotec
16	CD20	LT20.34	Miltenyi Biotec
17	CD24	32D12	Miltenyi Biotec
18	CD25	3G10	Miltenyi Biotec
19	CD29	TS2/16.2.1	Miltenyi Biotec
20	CD40	HB14	Miltenyi Biotec
21	CD41b	REA336	Miltenyi Biotec
22	CD42a	REA209	Miltenyi Biotec
23	CD44	DB105	Miltenyi Biotec
24	CD45	5B1	Miltenyi Biotec
25	CD49e	NKI-SAM1	Nordic-MUbio
26	CD56	REA196	Miltenyi Biotec
27	CD61	VI-PL2	BD Biosciences
28	CD62P	REA389	Miltenyi Biotec
29	CD69	FN50	Miltenyi Biotec
30	CD80	2D10.4.12	Miltenyi Biotec
31	CD83	HB15.206.8	Miltenyi Biotec
32	CD86	FM95	Miltenyi Biotec
33	CD95	DX2	Miltenyi Biotec
34	CD235a	REA175	Miltenyi Biotec
35	CD326	HEA125	Miltenyi Biotec
36	REA	REA293	Miltenyi Biotec
37	mIgG1	IS5-21F5	Miltenyi Biotec
38	mIgG2a	S43.10	Miltenyi Biotec
39	mIgG2b	IS6-11E5	Miltenyi Biotec



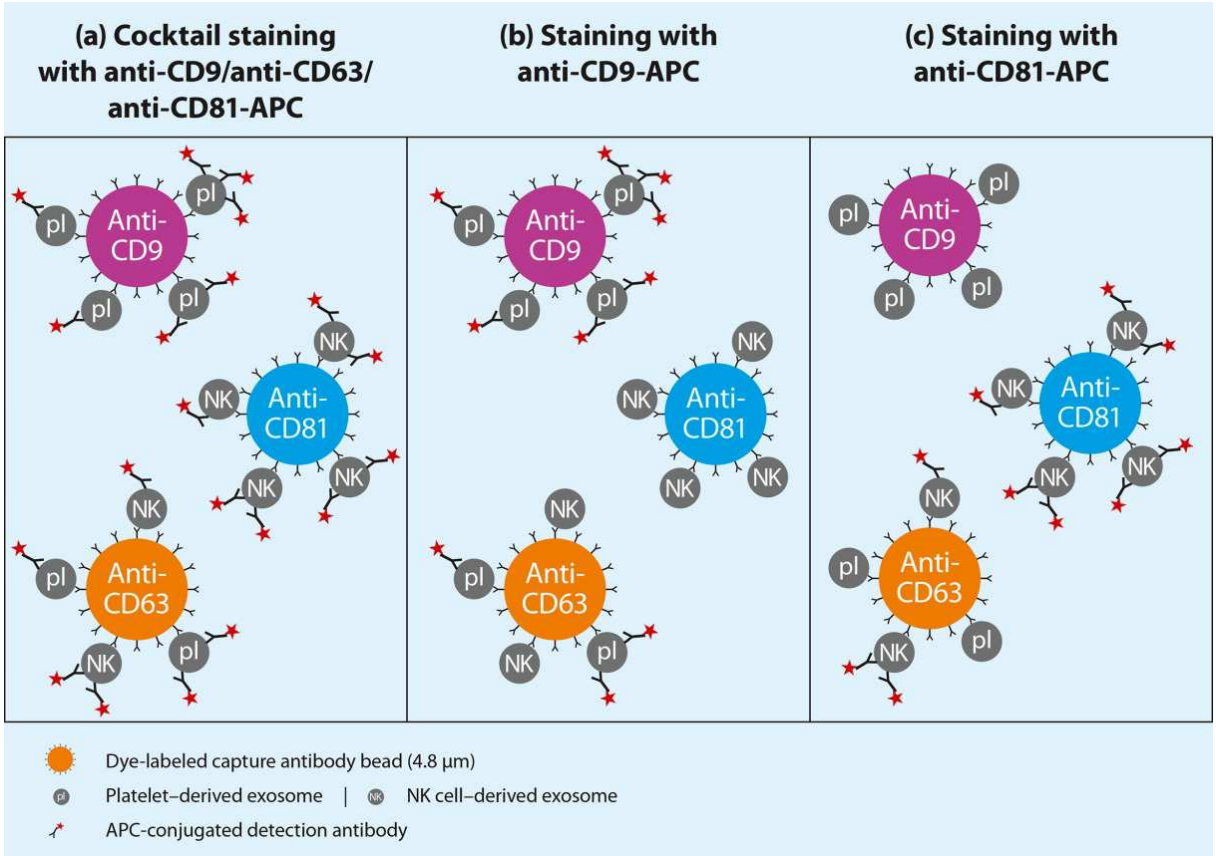
Supplementary figure 1: Specificity and bead set composition controls. **(a)** Median signal intensities after overnight incubation of capture antibody beads with 8 µg NK cell EVs (four donors) alone (circles) or in combination with an 100-fold excess of soluble mouse IgG1 isotype control (square) or soluble CD63 antibody (triangle) in relation to the capture antibody amount on the beads, followed by staining with anti-CD81-APC antibody for 1 h. **(b)** Median signal intensities after overnight incubation of 8 µg of NK cell EVs (four donors, D1-D4) with eight or 34 different capture antibody bead types and staining with a cocktail of anti-CD9-APC, anti-CD63-APC and anti-CD81-APC antibodies for 1 h. REA and mIgG1 indicate isotype control beads.



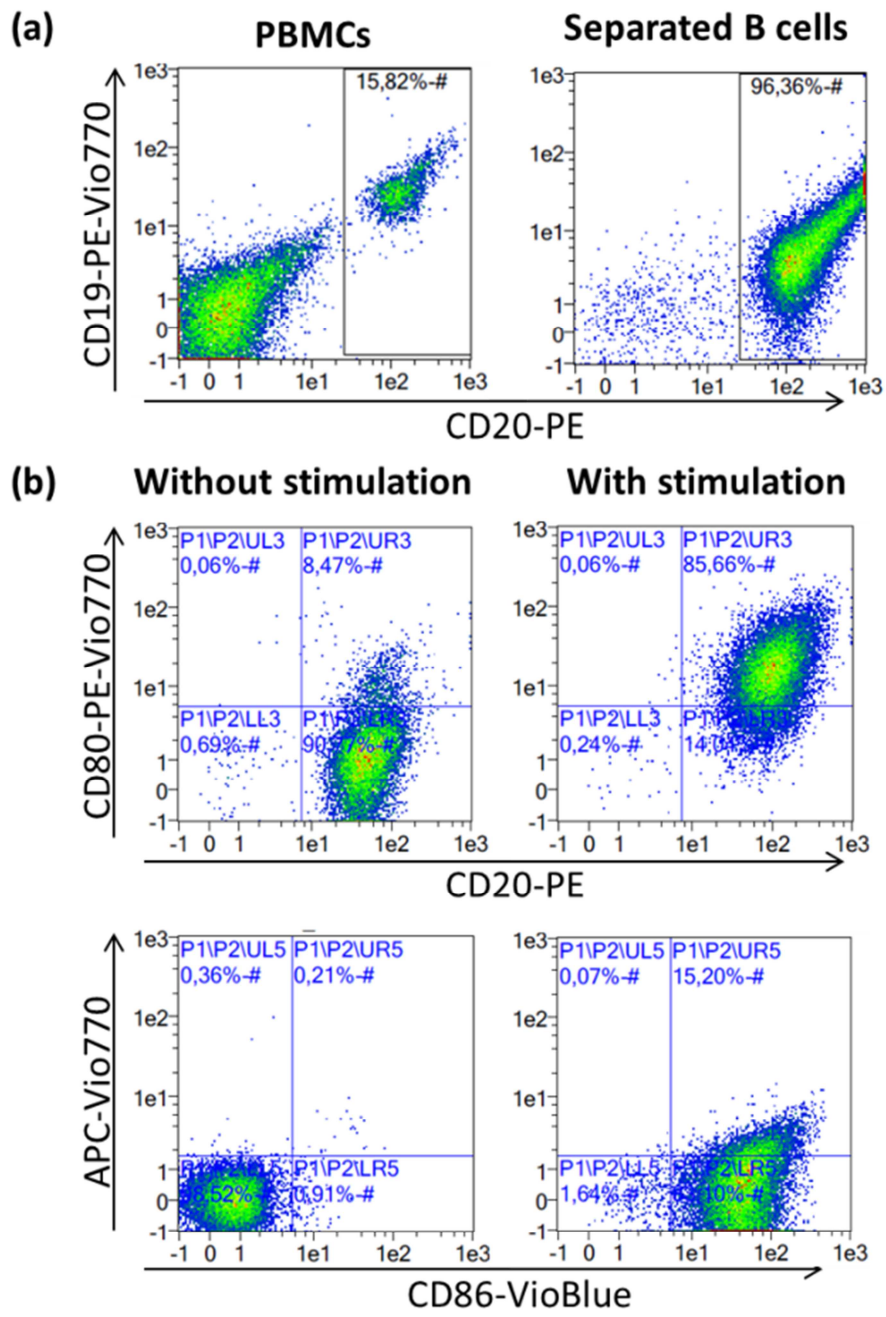
Supplementary figure 2: Repeated incubation of an EV sample with a 39-plex bead set. 8 µg NK cell-derived EVs were incubated with a 39-plex bead set overnight. The supernatant was incubated for a second capture with a fresh 39-plex bead set overnight. The bound EVs on both bead sets were stained with a cocktail of anti-CD9-APC, anti-CD63-APC and anti-CD81-APC antibodies for 1 h. REA and mIgG1 indicate isotype control beads.



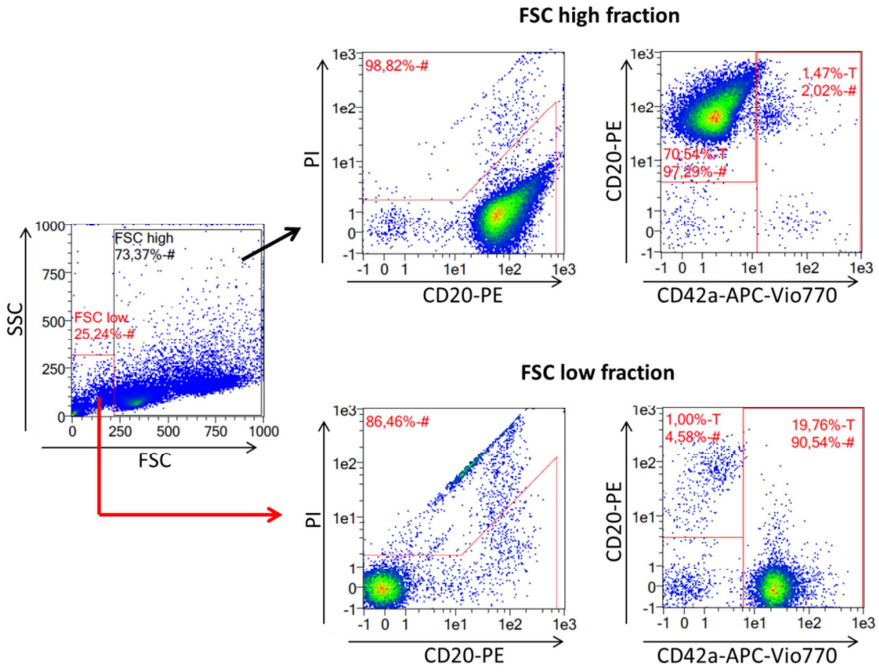
Supplementary figure 3: Nanoparticle Tracking Analyses (NTA) of EVs from **(a)** activated B cells and **(b)** stimulated platelets.



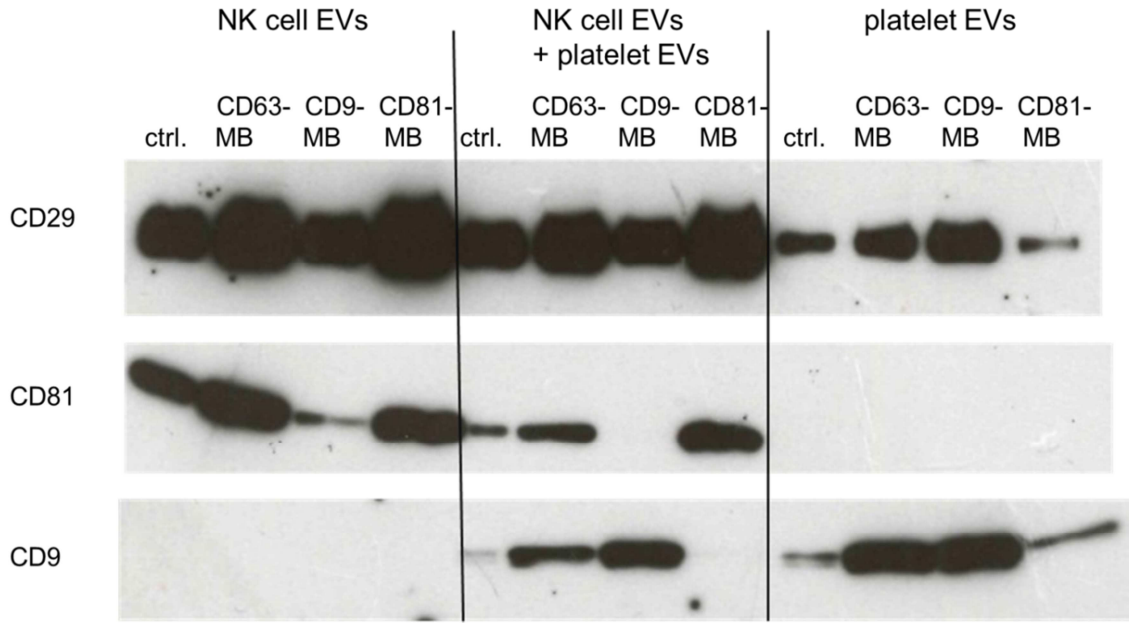
Supplementary figure 4: Schematic drawing of a mixture of $CD9^-/CD81^+$ NK cell EVs and $CD9^+/CD81^-$ platelet EVs on anti-CD9, anti-CD63, and anti-CD81-beads stained with **(a)** a cocktail of anti-CD9, anti-CD63, and anti-CD81-APC, **(b)** anti-CD9-APC, or **(c)** anti-CD81-APC.



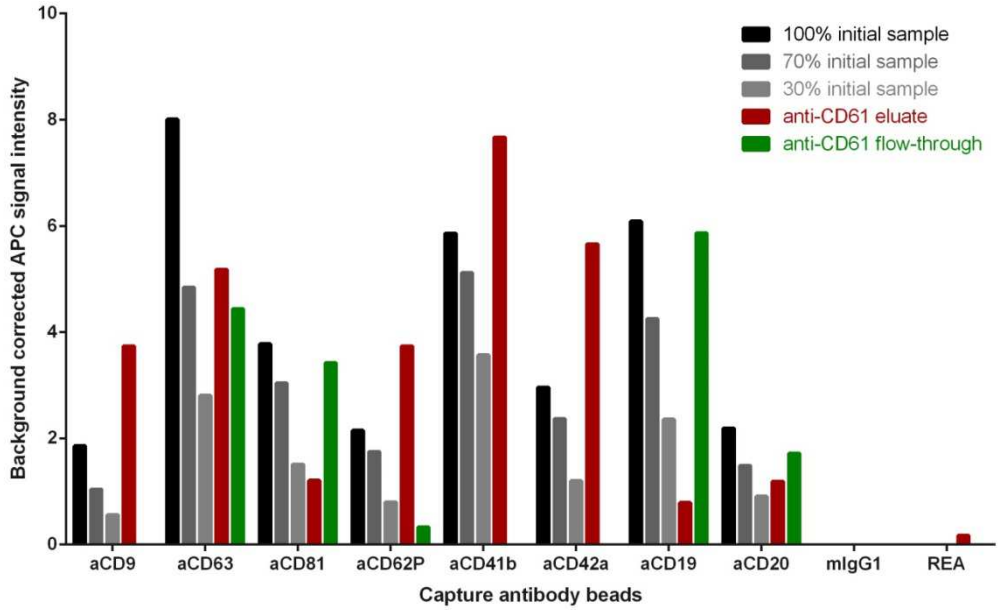
Supplementary figure 5: (a) Percentage of CD20-positive cells among PBMCs and after magnetic isolation of B cells. Debris and dead cells (PI-positive events) were excluded before B cell purity was determined. **(b)** Percentage of B cells that is positive for the B cell activation markers CD80 and CD86 after 5 days without and with stimulation with CD40 ligand and IL-4.



Supplementary figure 6: Flow cytometry analysis of separated B cells. The FSC high fraction that excludes potential debris (upper row) contains only 2% CD42a-positive events or 1.5% CD42a-positive events of the whole sample. But the FSC low fraction contains 90.5% CD42a-positive events or 19.8% CD42a-positive events of the whole sample. CD42a-positive events are most likely platelets.



Supplementary figure 7: Western Blot of NK cell EVs (left), a mixture of NK cell and platelet EVs (middle), and platelet EVs (right). Ctrl.: Loading control of 1.7 µg NK cell EVs, a mixture of 0.8 µg NK cell EVs and 1.7 µg platelet EVs, or 3.3 µg platelet EVs. CD63-MB, CD9-MB, CD81-MB: EVs isolated by CD63, CD9 or CD81 MicroBeads (25 µg NK cell EVs, a mixture of 12.5 µg NK cell EVs and 25 µg platelet EVs or 50 µg platelet EVs were used as starting material for magnetic sorting). CD29, CD81, and CD9: Detection antibodies.



Supplementary figure 8: Surface marker profile of EVs isolated from activated B cells before and after immunoaffinity isolation using anti-CD61 magnetic beads. Background corrected APC median signal intensities after incubation of 100%, 70% and 30% of the initial sample as well as the eluate and the flow-through after anti-CD61 bead separation with 39 capture antibody bead types and staining with a cocktail of anti-CD9-, anti-CD63-, and anti-CD81-APC antibodies. REA and mIgG1 indicate isotype control beads.